The Mitochondrial Toxin Ethidium Bromide Also Causes Genotoxicity in the Nucleus

by

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ABSTRACT

Mitochondria take part in many cellular events, such as ATP generation by oxidative phosphorylation, cellular signaling, and apoptosis. These organelles contain a genome (mtDNA), inherited from a bacterial progenitor, which plays a major role in mitochondrial function. Damage to mtDNA can cause disease and may play a causal role in aging. In this thesis, we have engaged in two studies related to the study of mtDNA using the budding yeast *Saccharomyces cerevisiae*.

Our major focus was on the cellular effects of the mitochondrial toxin ethidium bromide (EtBr). EtBr rapidly causes the destruction of mtDNA in *S. cerevisiae* and also engenders mtDNA loss in other organisms. For nearly fifty years, it has been suggested that EtBr solely affects mtDNA, leaving the nuclear DNA unscathed. However, we have now demonstrated that the effects of EtBr are, in fact, not limited to that organelle. We found that mutants lacking the ability to repair double-stranded DNA breaks in the nucleus are acutely sensitive to EtBr, even when mtDNA products or mtDNA itself are already lacking. We demonstrated that this toxic effect of EtBr in the nucleus causes an increase in nuclear genomic instability as measured by a novel assay of gene conversion. Our findings will have a major impact on the interpretation and design of experiments using this commonly used reagent.

In additional studies, we are seeking new proteins involved in mitochondrial division. Mitochondria constantly fuse and divide, and mutations perturbing mitochondrial dynamics affect the segregation and maintenance of mtDNA. We created a yeast strain that is inviable when mitochondria are able to divide. Subsequently, we are seeking genes that when overexpressed would potentially alter the stoichiometry or regulation of complexes involved in mitochondrial division, leading to a block in this process. I will discuss our progress so far in seeking novel mitochondrial division components.

ÖZET

Mitokondri, oksidatif fosforilasyon yoluyla ATP üretilmesi, hücre sinyallerinin iletilmesi ve apoptoz gibi birçok hücresel tepkimede görev alır. Bu organellerin bakteri olan atalarından kalan ve mitokondrinin işlevselliğinde önemli bir yere sahip genomları (mtDNA) vardır. Hasarlı mtDNA hastalıklara yol açabilir ve yaşlanmada önemli bir rolü olabilir. Bu tezde, tomurcuklanan maya *Saccharomyces cerevisiae*'yi kullanan mtDNA araştırmaları üzerine iki çalışma yer almaktadır.

Ana çalışmamız mitokondriyal toksin etidyum bromürün (EtBr) hücredeki etkileri üzerinedir. EtBr, *S. cerevisiae*'de mtDNA'nın hızlı yıkımına sebep olurken, diğer organizmalarda da mtDNA'nın kaybına sebebiyet vermektedir. Neredeyse 50 yıl boyunca, EtBr'nin sadece mtDNA'yı etkilediği, nükleer DNA'ya zarar vermediği düşünülmüştür. Ancak biz EtBr'nin etkilerinin aslında bir tek bu organelle sınırlı olmadığını gösterdik. Hücre çekirdeğindeki çift zincir DNA kırıklarının tamirini gerçekleştiremeyen mutantların, mtDNA'ya ya da mtDNA'nın kodladığı proteinlere sahip olmadığı halde, EtBr'ye duyarlı olduğunu bulduk. EtBr'nin hücre çekirdeğindeki toksik etkisinin, özgün gen dönüşümü testimizle ölçüldüğü üzere, genomik kararsızlıkta artışa neden olduğunu gösterdik. Bulgularımızın, yaygın olarak kullanılan bu kimyasalla yapılan deneylerin tasarım ve yorumlanmasında önemli bir etkisi olacaktır.

Ek çalışmalarda, mitokondrinin bölünmesinde görev alan yeni proteinleri aramaktayız. Mitokondri devamlı bölünmekte ve birleşmekte ve mitokondriyal dinamikleri bozan mutasyonlar mtDNA'nın kalıtımını ve korunmasını etkilemektedir. Mitokondri bölünebildiğinde yaşayamayan bir maya suşu yarattık. Akabinde, fazla ifade edildiğinde muhtemelen mitokondrinin bölünmesinde görev alan proteinin komplekslerinin stokiyometrilerini ya da regülasyonlarını bozarak, bölünmenin engellenmesine yol açacak genleri aradık. Mitokondriyal bölünmenin yeni bileşenlerini aramada geldiğimiz nokta da bu tezde yer almaktadır

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NOMENCLATURE

EtBr	Ethidium bromide
mtDNA	Mitochondrial DNA
nDNA	Nuclear DNA
gDNA	Genomic DNA
G418	Geneticin
CHX	Cycloheximide
5-FOA	5-Fluoroorotic acid
PCR	Polymerase chain reaction
DSB	Double strand break
YEPD	Yeast extract peptone dextrose
YPGE	Yeast extract peptone glycerol ethanol
SD	Synthetic dextrose
Ura	Uracil
Leu	Leucine
FALCOR	Fluctuation Analysis Calculator
MSS-MLE	Ma-Sandri-Sarkar Maximum Likelihood Estimator

Chapter 1

INTRODUCTION

Mitochondria are essential organelles that contain their own DNA. While mitochondrial DNA (mtDNA) is not essential for the cells to survive, mutations in mtDNA have been associated with neurodegenerative diseases as well as aging. Researchers deplete mtDNA from the eukaryotic cells to better understand the role of mtDNA in disease and aging. Ethidium bromide (EtBr), which is a DNA intercalating drug, is being used to destroy mitochondrial DNA in a diverse array of organisms, such as yeast, protists, flies, nematodes, as well as mammalian cells.

For decades, it has been assumed that EtBr does not affect nuclear DNA (nDNA) when used at concentrations that destroy mtDNA completely[1]. However, recent studies have suggested that EtBr could be toxic for nDNA. The first evidence of this toxic effect of EtBr came from the studies in the human pathogen- trypanosomes. EtBr was shown to be effective in killing trypanonosomes even when they lack mtDNA [2]. Another study which searched for yeast deletion mutants that are sensitive to EtBr treatment and consequent mtDNA loss identified mutants lacking the ability to repair nuclear double-stranded DNA breaks (DSB) as sensitive to EtBr [3]. However, using the microarray based growth measurement method that was used in this study, it was not possible to distinguish between whether the toxic effects of EtBr on mutants unable to engage in DSB repair resulted from the most immediate consequences of mtDNA loss or from direct effects of EtBr on nDNA replication. Actually, in a recent study, it was found that mitochondrial DNA maintanence is important for the cell cycle progression and and mtDNA loss evokes a DNA damage response.

In order to distinguish between the effect of EtBr and mtDNA loss, we separated the events of EtBr treatment and mtDNA loss by blocking function of mtDNA before EtBr treatment. For this purpose, we used yeast that are unable to replicate their mtDNA since they lack Mip1p, which is the polymerase responsible for the replication of mtDNA [4]. In this way we were able to deplete mtDNA from the cells without using EtBr. We also used an alternative way to block function of mtDNA. We knocked out *MRPL16* which encodes for a mitochondrial ribosomal protein. In this way, we deleted the mtDNA-encoded products from the cells. We showed that DSB repair system mutants were sensitive to EtBr even when they already lack mtDNA-encoded products or mtDNA itself. In the lack of mtDNA, nDNA is the only genetic material that could be affected by EtBr in the yeast that also lack DSB repair mutants. Therefore, in the first part of this thesis, we demonstrated that, contrary to previous reports, EtBr has a toxic effect in the nucleus. We also created a novel assay that can be used to distinguish between mutations happening at the single level or gross chromosomal rearrangements. Using this assay we showed that EtBr causes an increase in the gene conversion rate in the nucleus.

The second part of this thesis deals with mitochondrial dynamics. Mitochondria are dynamic organelles that continuously divide and fuse. When the mitochondrial fusion and fission are balanced; mitochondria display a tubular structure under the microscope. When the balance is distorted in favor of fusion, mitochondria become elongated, interconnected tubules; whereas they become small, fragmented DNA networks in case the balance shifts towards fission[5]. Defects in division or fusion are linked to neurodegenerative and neurodevelopmental diseases and aging in humans[6]. Budding yeast is a commonly used model organism to study mitochondrial dynamics, since the core machinery of mitochondrial division and fusion is conserved in humans and in budding yeast[7]. Although a number of proteins that take part in mitochondrial division and fusion have been identified, there are other parts of the machinery still waiting to be identified.

We conducted a novel screen to search for the unidentified components of fission. Our screen utilized a yeast strain that is inviable when mitochondria are able to divide. *fzo1Δ* causes the loss of mitochondrial DNA in the presence of mitochondrial fission, while *aac2Δ* mutants cannot grow in the absence of mitochondrial DNA. This strain, which lacks both Fzo1p and Aac2p, is transformed with a 2μ vector based high copy plasmid library. The 2μ origin of replication allows the overexpression of the genes that are carried in th plasmid. One of the possible outcomes of this transformation is over expression of a suppressor of *fzo1Δaac2Δ*. We have screened 40000 transformants by this method but we haven't yet found a suppressor of *fzo1*Δ*aac2*Δ.

Chapter 2 provides a background for the usage of EtBr, and a literature review on double strand break repair. The known components of mitochondrial division and fusion and the importance of the mitochondrial dynamics for the cell are also reviewed.

Chapter 3 includes the methodology used to show the effect of EtBr on nuclear DNA. Construction of the strain used to distinguish between mutations and gene conversions is also described. The chapter provides with the results that prove the effect of EtBr on nuclear DNA and proposes genomic instability as a possible mechanism for that effect. The chapter ends with a discussion of the results and suggests some possible directions that could be taken to elaborate the study.

Chapter 4 describes the screen conducted to search for an unidentified component of mitochondrial division machinery in *Saccharomyces cerevisiae.* After a brief introduction to mitochondrial dynamics, the methodology of the screen is explained.

The Appendix section summarizes our efforts to create a new method to distinguish between single nucleotide changes and genomic instability in single step. The construction of and possible uses of a new strain of yeast are explained. This new method is used to show the effects of the drugs that are already known as genomic instability causing agents. This section describes the methodology and discusses the efficiency of our method.

Chapter 2

LITERATURE REVIEW

2.1 Overview

Mitochondria are essential organelles that take part in a variety of processes including adenosine triphosphate (ATP) synthesis, ion homeostasis, cell fate determination, lipid metabolism and apoptosis. They are thought to be derived from ancient bacteria and early eukaryotes via endosymbiosis[8]. Mitochondria not only have their own genetic material in the form of deoxyribonucleic acid (DNA) but also they are able to synthesize some of the mitochondrial proteins via their own protein synthesizing systems.

Mutations in mitochondrial DNA (mtDNA) are related to a variety of diseases, including aging, cancer and neurodegenerative diseases in humans [9] [10]. *S. cerevisiae* is a good model organism to study mitochondrial disease since regulation of mitochondrial dynamics and mtDNA maintenance are conserved[11]. Other advantages of using budding yeast to study mitochondria include its tendency to accept the plasmids and/or gene fragments via transformation. For this reason, knocking out a gene in the budding yeast is convenient. Also, the doubling time of *S. cerevisiae* is only around 90 minues and it is easy to handle *S. cerevisiae* [12].

2.2 *mtDNA* is not essential for viability in S. cerevisiae

Mitochondria are essential organelles. Although mitochondria also have their own DNA, mtDNA is not essential for cell survival in some organisms such as the budding yeast. However, certain mutations in nuclear DNA (nDNA), which would be null mutations in cells having mtDNA, could kill a cell that does not have mtDNA. It has been found that mtDNA mutations accumulate with aging and in the case of neurodegenerative diseases

such as Alzheimer's [13]. Therefore mtDNA studies could help reveal the mechanisms and find cures, for these diseases. Yeast, whose mitochondrial biogenesis apparatuses are conserved, when compared to humans, can live without mtDNA. Yeast cells with destroyed mtDNA show a phenotype called "petite". The cytoplasmic petite phenotype, which is manifested by small and slow growing colonies of budding yeast unable to grow on non fermentable media [14], could be a result of total destruction of mtDNA or mutations in mtDNA [15]. The petite yeast first identified as distinctly small colonies that are formed on fermentable media. These yeast were respiratory deficient and the petite mutation was found out to be inherited in a non-Mendelian fashion. This segregation pattern implied that the petite phenotype was inherited via a cytoplasmic factor. This factor, which is called ρ, was first thought to be mitochondria. Sucrose density gradient experiments showed that the petite yeast, in fact, has mitochondria. However, they were missing some of the cytochromes that take part in respiration. More sophisticated gradient experiments that used an X-ray contrasting agent showed DNA associated with mitochondria. Later mtDNA was shown to be missing from petite mutants. Hence, the cytoplasmically inherited ρ factor was identified as mtDNA. The yeast with mtDNA are named as ρ^+ and the yeast lacking mtDNA are called ρ^0. DNA intercalating dyes such as ethidium bromide (EtBr), as well as chemicals interfering with DNA synthesis, have been used to induce petite mutations in budding yeast. Acriflavine was the first chemical that was shown to induce petite mutations. Since then cyanide, ethylene oxide, 2,3,5-triphenyltetrazoliumchloride, acridine derivatives, heavy metals, caffeine, enzyme inhibitors such as sodium azide and nitrophenols have been used, although with varying yields, to induce petite mutation. High temperatures can also induce petite phenotype[16].

Since its introduction as a petite mutation inducer, EtBr has been the most common agent used to destroy mtDNA because of its efficiency. EtBr destroys the mtDNA in 99.8% of cells in just 2-3 doublings. While using EtBr has become the gold standard for depleting

mtDNA from the cells, for many years, it has been suggested that EtBr does not have an effect on nuclear DNA (nDNA) at the concentrations used to destroy mtDNA [17] [18]. However, recent studies do imply a possible effect of EtBr on nDNA. An example of these studies was done on the *Trypanosoma brucei*. *T. brucei* has a special kind of mtDNA which is called kinetoplast DNA (kDNA). EtBr, due to its ability to destroy kDNA, has been used as a therapeutical agent against cattle disease, which is caused by *T. brucei*. A recent study found that EtBr was effective in killing these parasites even when they were lacking kDNA [2]. A study in the budding yeast further raised the possibility of the toxic effect of EtBr on nDNA. A genome-wide microarray-based search for petite negative mutants, which cannot live without mtDNA, showed double stranded break (DSB) repair mutants to be sensitive to EtBr [3]. However, in this study it was not investigated if this sensitivity was an immediate consequence of EBr treatment or a result of the following mtDNA loss. In this thesis study, we have investigated if the toxic effect of EtBr on DSB repair mutants is a result of EtBr treatment.

2.3 DNA repair systems help to preserve the integrity of the genome

DNA might become damaged due to intrinsic (metabolic) factors or polymerase errors or environmental factors such as UV light, ionizing radiation or base damaging chemicals. The damages in DNA could be chemical distortions in the structure of bases, incorporation of the wrong nucleotide during replication or single strand or double strand breaks (DSB). Since DNA damage could disturb genome integrity, various mechanisms have evolved to fix DNA damage. These mechanisms can be classified as direct reversal of the damage, removal of the wrongly incorporated base or DNA lesions and post-replication repair of DNA damage [19] [20]. The direct reversal of damage happens at the nucleotide level via the action of a single protein. Several enzymes in yeast take action in removing spontaneously occurring chemical modifications from the bases. One of these enzymes is a

photolyase which takes part in the removal of modifications that occur as a result of UV damage [21]. Also, a methyltransferase in yeast removes the methyl group from methylguanine which mispairs with thymidine and from methylthymidine which mispairs with guanine[22]. Mismatch repair removes the wrong base that was incorporated during replication, while base excision and nucleotide excision repair mechanisms excise damaged DNA. The post-replication repair pathways bypass the error during replication. They allow the replication to continue and generate a new error-free template strand. This new strand is then used by the excision repair systems to repair the damaged DNA accordingly. The bypass of the lesion could happen in two ways: by fork regression or by recombination. When replication stops at the damaged lesion, an Okazaki fragment at the downstream is generated and replication continues from that Okazaki fragment resulting in a gap at the daughter strand corresponding to the place where the replication stopped. This gap could be repaired by two mechanisms- fork regression or recombination. Fork regression results in an error prone repair whereas recombination results in an error free repair. The error free repair is the major repair pathway, especially for double strand break repairs (DSB) in which recombination occurs between the homologous strands of DNA. The homologous region of undamaged parental strand recombines into the gap in the opposite daughter strand leaving a gap in itself. This gap is filled with the action of ligase and polymerase using the intact daughter strand as a template. The error which remained at the parental strand where replication stopped is repaired by excision mechanisms after replication finishes [19]. Homologous recombination could happen during mitosis [23]. Mitotic recombination could be a result of DSB repair and could lead to chromosome rearrangements and loss of heterozygosity. Each of these events in the DNA could cause genomic instability. Since genomic instability is a hallmark of a cancer, measuring the genomic instability is important.

2.4 Mutation rate could be quantified using fluctuation analysis

Even before the structure of DNA was discovered, Luria and Delbrück described the fluctuation method to distinguish between adaptation and random mutations. After they observed that viable mutant colonies arise some time after antibiotic treatment in a bacterial culture, they investigated whether the colonies were already resistant to the antibiotic before the addition of the antibiotic due to spontaneous mutations or if the resistant colonies arose as a response to the selective pressure. If a large number of parallel bacteria cultures are established, treated with an antibiotic and allowed to divide for several generations, if the resistant bacteria emerge after antibiotic treatment due to selective pressure, one would expect that the frequency of mutations would be more or less the same among the parallel cultures and the variance in the number of resistant bacteria per the same total number of bacteria plated would not vary too much since the mutation will be introduced at the same generation. However, if the spontaneous mutations have created resistant bacteria even before the antibiotic was added to the cultures, the number of resistant bacteria in the cultures after antibiotic treatment would vary depending on the generation in which the mutation occurs. If the mutation occurs early, the number of mutants in the culture would be large since they increase logarithmically with each division while mutation that would occur spontaneously later in another culture would result n fewer resistant colonies. Therefore, even if the mean of the number of mutants might be the same as those that were adapted to the selective pressure of the antibiotic, the variance of the number of mutants would vary more when the mutations occur spontaneously. The problem with the calculation of actual mutation rates arises here: The resistant bacteria that could be counted are not the bacteria that were mutated; they are the offspring of the first mutated bacteria, whose number depend on how early the mutation occurred. Therefore a

simple division of the number of mutants to the total number of colonies in each culture would not give an accurate estimation of mutation rates [24 25 26] .

To calculate the probability distribution of mutation rates, Luria and Delbruck suggested two mathematical models. In the first method, using the definition of mutation rate as "the chance of mutation per bacterium per time unit", the fraction of the parallel cultures that do not have resistant individuals is used to calculate the rate of mutations, whereas, in the second method the mean number of mutants was the basis of the calculation [25,27].

These models have been improved by other mathematical models to obtain a more accurate distribution of mutation rates for all the rates and for all sample sizes. The "method of median" implemented by Lea and Coulson[28] is the most popular method to calculate the frequency of the mutations in a culture. The Ma-Sandri-Sarkar Maximum Likelihood Estimator (MSS-MLE) is also an accurate method of estimating the mutation rate [29,30]. Its power comes from the fact that it does not rely on the median of mutations when estimating the mutation rate but instead gives a better estimation using the entire dataset. The algorithm for MSS-MLE requires computational methods. Calculators for this method can be found at the FALCOR website[31] or solved using MATLAB. In this thesis a modified version of Lea-Coulson's method of median available at FALCOR website is used.

2.4. Defects in fusion causes mitochondria to lose mtDNA

Mitochondria are dynamic organelles that divide and fuse continuously. In a healthy cell, there is a balance between the fusion and fission of mitochondria. When this balance is conserved mitochondria can be seen as tubular structures under the microscope. The

tubular structure of mitochondria becomes distorted when the balance between fusion and fission is lost. When fusion is defective, mitochondria become small and fragmented whereas defects in fission cause mitochondria to appear as elongated, interconnected tubules. Interestingly, when both fission and fusion are inhibited, the tubular shape of mitochondria is restored [32].

Defects in mitochondrial dynamics have been associated with many diseases and disorders including Parkinson's disease, optic atrophy and several types of Charcot-Marie-Tooth disease [6]. Therefore understanding the fusion and fission machineries of mitochondria is of crucial importance. Several components of the fusion and fission machineries have already been identified. The core machinery of mitochondrial division and fusion is conserved in humans and in budding yeast. Especially, dynamin related proteins (Drp protein and Opa1p in humans and Dnm1p, Fzo1p, Mgm1p in yeast) are highly conserved. In yeast, Dnm1p is recruited to the surface of mitochondria by Fis1p via Mdv1p (Net2p) and Caf4p. In *dnm1Δ*, *fis1Δ* or *mdv1Δ* mutants, in which fission is distorted, mitochondria could not conserve their shape and they seem as net like tubular structures under the microscope. Moreover, Num1p and Mdm36p retain Dnm1p at the mitochondria surface as a separate mechanism and the connection of mitochondria to the cell cortex via Num1p and Mdm36p ensures the proper segregation and thus inheritance of the organelle [5]. Also, several different proteins take part in mitochondrial fusion. Fzo1p, which is a GTPase, is the protein that mediates mitochondrial membrane fusion whereas Mgm1p and Ugo1p are the other proteins that are essential for fusion [33]. Wild type yeast grows on both media containing fermentable substances such as dextrose (YEPD) and media containing non-fermentable substances such as glycerol and/or ethanol (YPGE). Distortion of fusion via deletion of the *FZO1* gene causes the production of fragmented mitochondria that lose their mitochondrial DNA. These mtDNA lacking mutants could not grow on non-fermentable media such as YPGE; whereas elongated mitochondria resulted

from deletion of *DNM1* retain their mtDNA and are able to grow on non fermentable media. However, the double mutants of *dnm1Δfzo1Δ* recover their mtDNA, so they regain their ability to grow on non-fermentable media [34]. This property of mitochondrial dynamics have been used for screens to find the components of fusion and fission machinery.

Mitochondrial carriers provide the movement of a number of small molecules such as adenine nucleotides, inorganic ions, amino acids, fatty acids across the inner membrane. The F_1Fo-ATP synthase synthesizes ATP within the mitochondrial matrix and this ATP is transported out of the inner membrane by the ADP/ATP carrier (AAC) proteins. This export of synthesized ATP is coupled to the import of ADP into the mitochondrial matrix. An electrochemical gradient, which is coupled to oxidative phosphorylation, is generated during the movement of protons into the intermembrane space. The transmembrane potential that is created by the electrochemical gradient should be maintained in the cell to survive. The proton motive force is the main energy that releases ATP from its binding site in ATP synthase. Aac2 protein is the major mitochondrial ADP/ATP translocator. Some subunits of electron transport chain and F_1Fo ATP synthase are encoded by mitochondrial genome[35]. The translocation of ADP and ATP by Aac2p is required for viability of cytoplasmic petites which are yeasts lacking mtDNA[36]. When *AAC2* is deleted in cells lacking mtDNA, the cells fail to maintain the mitochondrial membrane potential and die.

Chapter 3

Toxic Effect of Ethidium Bromide on Nuclear DNA

3.1 Introduction

Mitochondria have evolved from protobacteria via endosymbiosis. They retained their DNA but lost their ability to live independently as the mtDNA started to be controlled by the nuclear genome. Mitochondria has become essential for the eukaryotic cells. However, mtDNA is not essential for some organisms such as the budding yeast *S. cerevisiae*. The cells that lost their mtDNA are called "cytoplasmic petites". EtBr has been used to deplete mtDNA not only from yeast but also flies, nematodes, mammals. For decades, EtBr was suggested not to have a toxic effect on nuclear DNA at the concentration that is being used to deplete mtDNA. However, recent studies suggested possible effects of EtBr outside of mtDNA. EtBr was found out to be effective in killing trypanosomes that lack a special form of mtDNA, which is kinetoplast DNA [2]. Moreover, a microarray-based screen for the yeast mutants that are unviable on medium containing EtBr revealed a number of nuclear DNA repair mutants including *rad57*Δ, *mus81*Δ, *mms1*Δ, *mms4*Δ, *xrs2*Δ, *rad54*Δ, *rad53*Δ, and *rad55*Δ mutants as sensitive to EtBr[3]. However, it was unclear if this sensitivity was a result of EtBr treatment or the mtDNA loss that follows EtBr treatment. In this part of my thesis, my aim was to understand if EtBr affects the nDNA directly. To understand the direct effect of EtBr on nDNA we first deleted mtDNA-encoded products in the cell by knocking out *MRPL16*, a gene that codes for a subunit of mitochondrial ribosome. Although it has been known that mtDNA has to be expressed in order to keep itself intact in W303 background [37] , mtDNA was not totally lost in Mrpl16p lacking yeast in the generic BY background of yeast. A recent study used *MGM101* deletion to deplete mtDNA from yeast cells[38]. Mgm101p is a protein that takes part in mtDNA repair. The

Mgm101p-lacking cells do not have intact mtDNA. We used a similar approach and in order to deplete mtDNA from cells without using EtBr, we knocked out *MIP1*, which codes for the catalytic subunit of the only known mitochondrial polymerase. Mip1p-lacking cells are not able to replicate their mtDNA which will eventually cause mtDNA depletion.

3.2 Results

3.2.1 Double strand break repair mutants are sensitive to EtBr

Rad51p and Mre11p are two of the many enzymes that take part in the repair of the double strand breaks within nuclear DNA. We observed that the growth of the mutants that lack either of these enzymes is more diminished when compared to the wild type, when they were grown on EtBr containing media (Fig 3.1a). This effect on growth was retained even when the cells were lacking mitochondrial DNA encoded products (Fig 3.1b) or mitochondrial DNA polymerase (Fig 3.1c). The effect of EtBr was more severe for the *mre11*Δ mutants than the *rad51*Δ mutants. This could be explained by the fact that Rad51p has orthologs such as Rad55p and Rad57p which can compensate for its loss, whereas Mre11p does not. http://www.ncbi.nlm.nih.gov/pubmed/7705645/

Since the spot assay gives only a semi quantitative result we determined the effect of EtBr on the proliferation of yeast by calculating the doubling time for the wild type (BY4742), yeast lacking Mrpl16p (CDD429), Mre11p (CDD263) and Rad51p (CDD262), as well as the double mutants *mrpl16*Δ*mre11*Δ (CDD431), *mrpl16*Δ*rad51*Δ (CDD430). When the doubling time for each of these strains in the absence or presence of 25 μg/ml EtBr were calculated, it was seen that EtBr caused only a 4% increase in the doubling time of the yeast that lacked mtDNA encoded products. However, when the yeast lack DSB break repair enzymes, the proliferation rate decreased 29% for *rad51*Δ mutants and 37% for *mre11*Δ mutants when they grew in the presence of EtBr. If the yeast lacks mtDNA

encoded products in addition to DSB repair enzymes, EtBr treatment causes a 33% decrease in the proliferation rate of *mrpl16*Δ*rad51*Δ and 25% decrease in the proliferation rate of *mrpl16*Δ*mre11*Δ (Fig 3.2b). A decrease in the proliferation rate is also observed for *mip1*Δ*mre11*Δ (Fig 3.2c).

3.2.2 EtBr does not cause an elevated rate of mutation at *CAN1* locus

After showing that the effect of EtBr on nucleus is direct we wanted to show the magnitude of that effect. Does EtBr increase the rate of mutation at a single gene level or does it cause gross chromosomal rearrangements? As mutagenesis at the level of single genes might be one of the possible ways that EtBr affects nDNA we used fluctuation analysis to calculate mutation rates. We looked at the mutation rate of the *CAN1* gene when the cells were treated or not treated with EtBr. *CAN1* gene in yeast codes for arginine permease. Mutations in that gene results in defects in arginine uptake. Canavanine is an arginine analog and toxic to cells when uptaken via arginine permease action. When *CAN1* gene is mutated the permeability of the cell to arginine and its analogs is blocked. Therefore this mutation results in resistance to canavanine. Therefore we used the number of surviving colonies on a plate that contains canavanine and arginine as the number of mutants that is used for fluctuation analysis.

From Table 3.1 it can be seen that the rate of mutations in the *CAN1* locus do not change significantly upon EtBr treatment in the yeast lacking mtDNA-encoded products. This result shows that EtBr does not increase mutation rates at the single gene level.

3.2.3 Gene conversion rate increases with increasing concentrations of EtBr

We have treated the cells lacking *MIP1* with different concentrations of EtBr, including 25 μg/ml, which is the standard concentration that is being used to destroy

mtDNA. The *CYH2* gene, which encodes for a protein of the ribosomal large subunit, confers cycloheximide (CHX) resistance when mutated. The mutant allele is recessive for drug resistance in diploids. We have created diploid yeast strain that has the *cyh2* mutation in one chromosome and has a tightly linked antibotic G418 resistance gene, *KanMX4, in trans* to the *cyh2* allele. This strain is cycloheximide sensitive and G418 resistant. It could become cycloheximide resistant either by mutation or by gene conversion. If it is a mutation, then only the *CYH2* gene is affected and the cell will retain its resistance to G418 while becoming resistant to CHX. However, if the cause of the resistance to cycloheximide is the recombination of the recessive resistant allele, then the G418 resistance marker will be recombined to the other chromosome, too. In this case, the yeast will become sensitive to G418 while becoming resistant to CHX. I used the number of colonies that survives in CHX containing plates after being grown in the presence or absence of EtBr and dies when replica plated to G418 containing plates to calculate the gene conversion rate; and the number of colonies that survived in both G418 containing plates and CHX containing plates to calculate the point mutation rate via fluctuation analysis (Figure 3.3).

We have shown that the gene conversion rate in Mip1p lacking cells increases with the increasing amounts of EtBr. The gene conversion rate is increased to 2.4 fold of untreated cells when treated with 25 μg/ml EtBr, and to 3.7 fold when treated with 50 μg/ml EtBr. These results were obtained using 14 cultures in total. Although another increase was observed for the cultures that were grown in 100 μg/ml EtBr, the variance was too high. Therefore it is difficult to compare the samples treated with 100 μg/ml EtBr with the samples that were treated with less amounts of EtBr. This variance is most likely due to the fact that the sample size is limited to 7 since the cells have a tendency to not proliferate in 100 μg/ml EtBr. Another interesting observation from these results was that the increase in the gene conversion rate in the cultures that were treated with hydroxyurea (HU), a chemical that is known to cause genomic instability by depleting nucleotides from the

environment, was limited to only 2.2 fold of the gene conversion rate of non-treated cultures (Figure 3.4).

We have also investigated the gene conversion rate in yeast lacking mtDNA-encoded products. Our strain which has an antibiotic resistance gene (*KanMX4*) linked *in trans* to the recessive *cyh2* allele enabled us to distinguish whether cycloheximide (CHX) resistance is conferred by mutation at the single gene level or by chromosomal rearrangement.The fluctuation analysis shows that both mutation rate and gene conversion rate increases with the increasing amounts of EtBr. The data sets for the Figures 3.5a and 3.5b are combined from three independent assays consisting of 5,5 and 4 parallel cultures. For fluctutation analysis, they were treated as a one 14 parallel cultures experiment.

3.2.4 EtBr concentrations under 5 µg/ml minimally affect the growth rate of cells lacking both intact mtDNA and DSB repair

In an effort to find a concentration of EtBr that would not interfere with the nucleus in the experiments where EtBr is used to deplete mtDNA, we searched for the concentration of EtBr that does not cause a growth deficiency for the cells lacking both mtDNA and DSB repair (*mrpl16*Δ*mre11*Δ). We detected that 5 µg/ml EtBr minimally affected the growth rate of these cells (Fig 3.6a). Concentrations less than that also did not have any significant effect on the growth of cells lacking both mtDNA encoded products and DSB repair (Fig 3.6b). 5 µg/ml EtBr also has only small effect on the growth of cells lacking mitochondrial DNA polymerase and DSB repair (Fig 3.6c).

3.2.5 3 µg/ml or more EtBr provides >99% mtDNA loss

Yeast lacking the ATP/ADP transporter Aac2p cannot grow in the absence of mtDNA. We used this fact to detect a minimum concentration of EtBr that could let us

remove mtDNA from the cell without affecting the nuclear DNA. We observed that 5 μg/ml EtBr was enough to remove more than 99% of the mtDNA. Concentrations more than 5 μg/ml EtBr showed a similar effect (Fig 3.7a). In a further effort to detect the minimum concentration of EtBr that provides total mtDNA loss more precisely, we checked the survival rates of *aac2*Δ mutants in EtBr concentrations less than 5 μg/ml. While 1 and 2 μg/ml EtBr provided mtDNA loss to some extent, 3 μg/ml EtBr was enough to kill more than 99.9% of the cells that cannot grow without mtDNA. Therefore, we can say that 3 μg/ml EtBr is the minimal concentration that will allow a total mtDNA loss (Fig 3.7b). As the effect of EtBr, at concentrations less than or equal to 3 μg/ml, on the growth of yeast lacking DSB repair proteins is negligible when compared to that of 25 μg/ml EtBr, we would suggest the researchers to use 3 μg/ml EtBr instead of 25 μg/ml EtBr to remove mtDNA from the cells to avoid effect on nDNA.

3.3 Discussion

In this study we have shown that the sensitivity of DNA DSB repair mutants to EtBr is due to the direct effect of EtBr on nuclear DNA. We demonstrated that this effect is increasing the rate of mitotic recombination, which causes genomic instability. We found out that the effect of EtBr on growth of DSB repair mutants without an intact mtDNA is minimal when EtBr is used at concentrations lower than 5 μg/ml. Moreover, mtDNA in 99.9% of cells were removed when the cultures were treated with EtBr at concentration equal to or more than 3 μg/ml. Therefore, we suggest using 3 μg/ml EtBr to deplete mtDNA in order to avoid any possible interferences of EtBr with the results of the study.

As we already know from the previous study of Dunn et al. [3] the DSB repair mutants are sensitive to EtBr. We showed that this sensitivity is retained even when the mtDNA is not intact. A decrease at the proliferation rate of DSB repair mutants is observed upon EtBr treatment regardless of whether they have intact mtDNA or not. This implies

that the toxic effect of EtBr on nuclear DNA could be fixed by the DSB repair mutants. This is most likely why the effect of EtBr on nDNA was overlooked for decades. Although it is fixed through DSB repair, the effect of EtBr still should be taken into consideration especially when the gene or protein expression levels of EtBr-generated ρ^0 cells are being investigated.

As the DSB repair proteins are needed to recover from the toxic effects of EtBr, we speculate that EtBr causes replication fork pause and/or collapse upon intercalating into DNA. Actually, this could be tested using 2D gel electrophoresis. If the replication fork pauses, replication intermediates will accumulate and will form an accumulated spot on the 2D gel[40].

We have used the cycloheximide resistance mutator phenotype as an indicator of the changes that EtBr causes on DNA. The strain we have designed gives us the ability to detect if the resistance is due to a mutation or a recombination. The diploid strain has an antibiotic resistance marker *in trans* tightly linked to the recessive cycloheximide resistant allele. In case of a recombination the antibiotic resistance marker will be recombined along with the cycloheximide resistant allele. Therefore, the yeast will lose its resistance to the antibiotic. However, a mutation causes the cycloheximide resistance, the antibiotic resistance marker would not be affected. Therefore, the yeast will be resistant to both cyloheximide and the antibiotic. This system, with little modification, could be used to detect the genotoxicity of chemicals and pharmaceuticals. An attempt to create a strain that could be used to detect the chromosomal recombination in one step with counter-selection is explained in the Appendix section.

An interesting observation is that the gene conversion level does not change dramatically upon HU treatment when compared to the increase of recombination rate upon EtBr treatment. Actually, a more dramatic increase in gene conversion levels is expected after HU treatment since HU is an inducer of genomic instability. This implies that the

mechanisms through which they cause genomic instability are different for HU and EtBr. HU depletes the nucleotides from the cells. This depletion causes replication fork stalling.

Lastly, the sensitivity of DSB repair mutants to EtBr suggests that genetic screens using EtBr could be conducted to identify novel proteins that could be related to DSB repair.

3.4 Materials and Methods

3.4.1 Strains, media, and genetic methods. A list of the strains that are used in this study can be found in Table (3.3) along with their genotypes. A list of primers used in this study can be found in Table (3.4).

The BY4742 strain is a derivation of the S288C strain [41] and it was bought from EUROSCARF. CDD262 (*rad51*Δ) and CDD263 (*mre11*Δ) were also bought from EUROSCARF and they are confirmed by barcode sequencing using primers 3 and 74 whose sequences could be found at Table (3.3). CDD409 is the spore of the strain obtained knocking out mrpl16 by URA3 which was amplified from pRS306 with primers 260 and 261. CDD409 was crossed to CDD262, sporulated and spore *rad51*Δ*::KanMX4 mrpl16*Δ*::URA3* was named CDD430. CDD409 was crossed to CDD263, sporulated and spore *mrpl16*Δ*::URA3* was named CDD429, spore *mre11*Δ*::KanMX4* was named CDD431.

The *mrpl16*Δ strain that was used for the canavanine resistance mutation assay was obtained by the spore that was obtained after sporulation of the mating of BY4742 with CDD409. This strain is named CDD501.

CDD442, in which *ygl104c* was marked with *KanMX4* which is tightly linked to *CYH2*, was bought from EUROSCARF. CDD442 and CDD409 were mated and sporulated.

CDD65 was made by transferring mutant *cyh2* gene from CDD51, which is obtained from the wild type in W303 background in the presence of 10mg/L cycloheximide, to BY4743. CDD307, a strain that lacks both *URA3* and *KanMX4* markers, is used to *mrpl16*Δ strain CDD474. *MRPL16* was knocked out by *LEU2* which was amplified from pRS305 with primers 260 and 261 in CDD307 and sporulated. The resulting strains is named as CDD474. The spores of CDD442 x CDD409 mating and the spores of CDD65 x CDD474 mating were mated to obtain the diploid *mrpl16*Δ*::LEU2/mrpl16*Δ*::URA3 cyh2/CYH2 vps73*Δ*::KanMX4/VPS73* strain CDD502 that we used in gene conversion assay.

BY4741 and CDD263 were crossed. *MIP1* in the yeast from that cross was knocked out by *URA3* which was amplified from pRS306 with primers 434 and 435 and the yeast sporulated.The *mip1*Δ*::URA3* spore was named as CDD623 and the *mip1*Δ*::URA3 mre11*Δ*::KanMX4* strain was named as CDD624.

The *MIP1* lacking version of the strain that is used in gene conversion is obtained from the mating of a spore of CDD65 x CDD622 cross with a spore of CDD422 x CDD623 cross. The genotype of the resulting CDD641 strain is *vps73*Δ*::KanMX4/cyh2 mip1*Δ*::URA3/mip1*Δ*::URA3 met15*Δ*0/MET15 LYS2/LYS2 trp1*Δ*/TRP1.*

AAC2 lacking CDD68 is used to detect the mtDNA loss since it cannot live without mtDNA.

3.4.2 Serial dilution assay.

The cultures were grown to logarithmic phase of growth, and then diluted to 0.02 OD_{600}. Additional three five-fold dilutions are done and 4 ul of each dilution was spotted on YEPD and YEPD+ 25 μg/ml EtBr plates. The pictures of the plates were taken using Colony Doc-It system (UVP,Upland,CA) after incubation at 30°C for 3 days.

3.4.3 Measurement of proliferation rate.

The strains were grown in YEPD for overnight. In the morning, the cultures were diluted to 0.05 at OD_{600} in YEPD containing indicated concentrations of EtBr. Only when measuring the growth rate of CDD431, the cultures diluted to 0.05 OD_{600} were grown overnight. In the morning, they are once more diluted to 0.1 OD_{600} and their growth rate was tracked after that. This was done since CDD431 grows too slow which makes tracking its growth within a reasonable time period hard. Their OD at 600 nm is measured at 2-3 hour intervals using Mecasys Optizen POP UV-VIS (Mecasys Co. Ltd., Daejeon, South Korea).

The doubling time for each strain in each experiment was calculated in Microsoft Excel 2011 as the reciprocal of the slope of $\log_2$(OD_{600} measurements) vs. time graph. The experiment was repeated 3 times.

3.4.4 Canavanine resistance mutation assay.

Fresh CDD333 and CDD409 individual colonies were streaked onto YEPD and grown at 30°C for 3 days. Ten randomly selected CDD333 and CDD409 colonies were grown overnight at 30°C in YEPD. Each individual overnight culture were diluted to an OD_{600} of $1/ 2^{15}$ and vegetatively grown in YEPD in the absence of EtBr for 15 generations. CDD409 cells were also vegetatively grown in YEPD in the presence of 25 μg/ml EtBr for 15 generations. When the OD_{600} of the cultures became around 1.00, 200 ul aliquots were spread on SMM plates containing 60 μg/ml canavanine, and the plates were incubated at 30°C for 3 days before the canavanine-resistant colonies were counted using Colony Doc-It system (UVP,Upland,CA). The mutation rate is calculated using Lea-Coulson method of median available on Fluctuation Analysis Calculator (FALCOR) website based on Luria-Delbrück fluctuation analysis. The number of colonies on YEPD was used as the total number of cells whereas r values were the number of CAN^r colonies for each individual

culture. The same procedure is utilized for BY4742 and CDD263 to validate our procedure. n=10 for BY4742, CDD409 +/-EtBr, n=9 for CDD263 and CDD333.

3.4.5 Point mutation and gene conversion assay.

Ten CDD502 colonies, which were grown on YEPD for 3 days, were grown overnight in YEPD liquid culture. The overnight cultures were diluted to an OD_{600} of $1/ 2^{15}$ and were vegetatively grown in YEPD in the presence of 0, 25, 50 and 100 μg/ml EtBr for 15 generations. 200 μl aliquots were spread on YEPD and YEPD+CHX plates containing 10 μg/ml CHX with 1/10 and $1/10^4$ dilutions, respectively. The plates were incubated at 30°C for 3 d, and the cycloheximide-resistant colonies were counted using Colony Doc-It system (UVP,Upland,CA). After 3 days the colonies were replica plated to YEPD+G418 plates containing 200 μg/ml G418, and the G418 resistant colonies were counted the next day. The mutation rate is calculated using Ma-Sandri-Sarkar Maximum Likelihood Estimation (MSS-MLE) method available on Fluctuation Analysis Calculator (FALCOR) website based on Luria- Delbrück fluctuation analysis. The number of cells grown on YEPD is used as total number of cells for each sample. The r values are the number of cells that grew on CHX but not on G418 for gene conversion rate calculation, number of CHX resistant colonies for mutation rate calculation.

The same procedure was repeated for three times with 5 random parallel cultures of CDD641 so that the total number of cultures that is used to calculate the gene conversion and mutation rates is 15. 5 parallel cultures were vegetatively grown also in YEPD+ 20 mM HU for 15 generations as a control to the experiments with EtBr.

3.4.6 Determining the mtDNA loss at different concentrations of EtBr.

Overnight CDD68 (*aac2*Δ) cultures were diluted to 0.1 OD_{600} in YEPD containing EtBr at concentrations indicated at Table 3.2. They were allowed to grow at 30°C for 4 generations. 150 µl of the cultures with appropriate dilutions were spreaded on YEPD and YPGE. The cells were counted after incubation at 30°C for 3 days.

Figure Legends

Figure 3.1 (a) Yeast with defective DSB repair are more sensitive to EtBr. *rad51Δ* (CDD262) and *mre11Δ* (CDD263) cells, along with the isogenic wild-type strain (BY4742), were grown in YEPD broth for four generations at 30°C, then diluted to an OD_{600} of 0.02. Three further 5-fold serial were performed, and 4 µl of aliquots of each dilution were applied to the YEPD plates lacking (-EtBr) or containing (+EtBr) 25 µ/ml of drug. The plates were incubated in 30°C for 3 days. (b) *rad51Δ* and *mre11Δ* mutants retain sensitivity to EtBr, even when they lack mtDNA encoded products. *rad51Δmrpl16*Δ (CDD430) cells, *mre11Δmrpl16*Δ (CDD431) cells, and the isogenic *mrpl16*Δ (CDD429) control were treated as in (a). (c) *mre11Δ* mutants retain sensitivity to EtBr, even when they lack mtDNA polymerase. CDD624 (*mre11Δmip1*Δ*)* cells , and the isogenic CDD622 (*mip1*Δ) control were treated as in (a).

Figure 3.2 (a)The proliferation rate of yeast lacking DSB repair proteins decrease upon EtBr treatment. The proliferaton of log phase cultures of strains BY4742 (WT), CDD262 (*mre11*Δ*)*, CDD263 (*rad51*Δ*)*, are tracked spectrophometrically with 2-3 hour intervals. The doubling time calculated as the reciprocal of the slope of $\log_2$(OD_{600} measurements) vs. time graph. ($P < 0.05$ by two-tailed Student's t-test, unequal variance; $n = 3$; error bars relate standard deviation.) (b) *mre11*Δ mutants retain sensitive to EtBr when they lack

mtDNA-encoded products. The proliferation of strains CDD429 (*mrpl16Δ),* CDD431 (*mre11Δmrpl16Δ),* CDD430 (*rad51Δmrpl16Δ)* are tracked as in (a). ($P < 0.05$ by two-tailed Student's t-test, unequal variance; n = 3; error bars relate standard deviation.) (c) *mre11Δ* mutants retain sensitive to EtBr when they lack mtDNA. The proliferation of strains CDD2 (wild type), CDD262 (*mre11Δ*), CDD622 *(mip1Δ)* and CDD624 (*mre11Δmip1Δ)* are tracked as in (a).

Figure 3.3 An antibiotic resistance gene (*KanMX4*) linked *in trans* to the recessive *cyh2* allele enables us to distinguish whether cycloheximide (CHX) resistance is conferred by mutation at the single gene level or by chromosomal rearrangement. The strain becomes CHX resistant G418 sensitive in case of chromosal rearrangement whereas becomes CHX and G418 resistant in case of a mutation.

Figure 3.4 (a) (b) Both point mutations and gene conversion are increased by EtBr, even when mtDNA is not intact. Ten CDD 641 colonies were vegetatively grown in YEPD in the presence of EtBr for 15 generations. 200 µl aliquots were spread on YEPD+CHX plates containing 10µg/ml CHX, and the plates were incubated at 30°C for 3 days, and the cycloheximide-resistant colonies were counted. After 3 days the colonies were replica plated to YEPD+G418 plates containing 200 µg/ml CHX, and the G418 resistant colonies were counted the next day. The mutation and recombination rates are calculated using the modified Lea-Coulson method of median available on Fluctuation Analysis Calculator (FALCOR) website based on Luria- Delbrück fluctuation analysis. (n=14). The error bars represent the 95% confidence interval (CI).

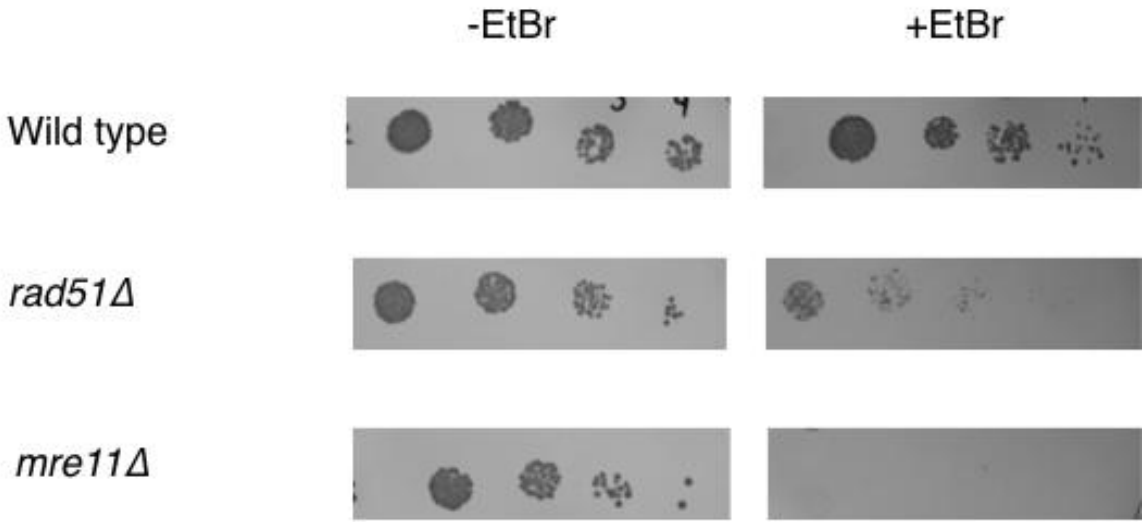
Figure 3.5 Both point mutations and gene conversion are increased by EtBr, even in the absence of mtDNA-encoded products. Ten CDD 502 colonies were vegetatively grown in YEPD in the presence of EtBr for 15 generations. 200 µl aliquots were spread on

YEPD+CHX plates containing 10μg/ml CHX, and the plates were incubated at 30°C for 3 d, and the cycloheximide-resistant colonies were counted. After 3 days the colonies were replica plated to YEPD+G418 plates containing 200 μg/ml CHX, and the G418 resistant colonies were counted the next day. The mutation rate is calculated using Ma-Sandri-Sarkar Maximum Likelihood Estimation (MSS-MLE) method available on Fluctuation Analysis Calculator (FALCOR) website based on Luria- Delbrück fluctuation analysis. (n=9) The error bars represent the 95% confidence interval (CI).

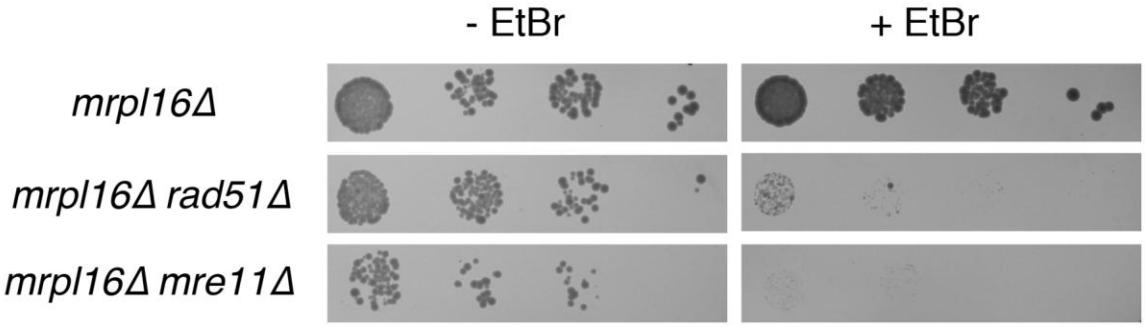
Figure 3.6 (a) CDD 431 **(***mre11Δmrpl16*Δ*)* cells were diluted into YEPD medium containing the indicated amount of EtBr, and the growth rates of cultures were spectrophotometrically monitored at 600 nm. The doubling times calculated as the reciprocal of the slope of log2 (OD_{600} measurements) vs. time graph. (b) The same procedure as (a) is done for *mre11Δmip1*Δ cells. (n=3). The error bars represent standard deviation.

Figure 3.1 Effect of EtBr on the growth of DSB repair mutants in the presence and absence of mtDNA encoded products

a.



b.



c.

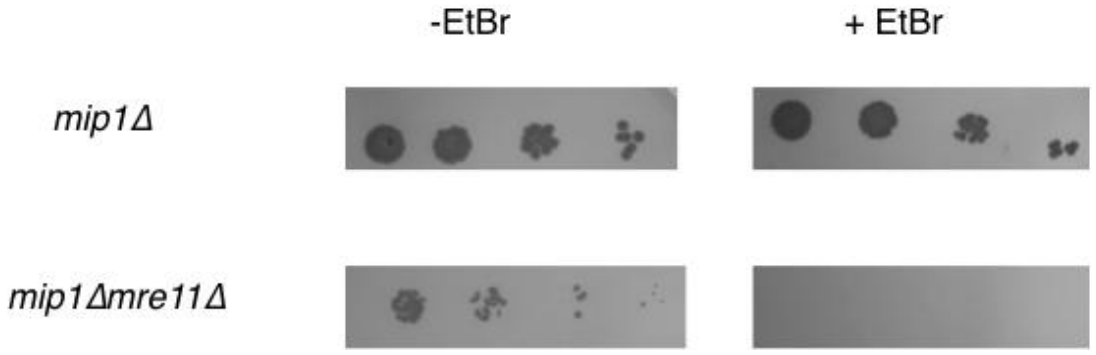
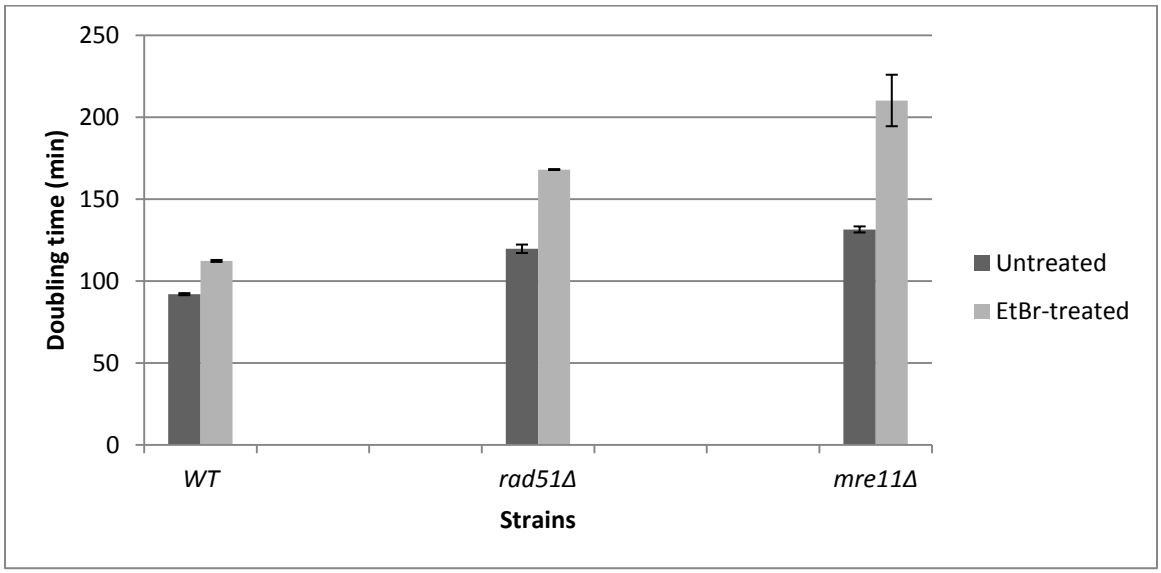
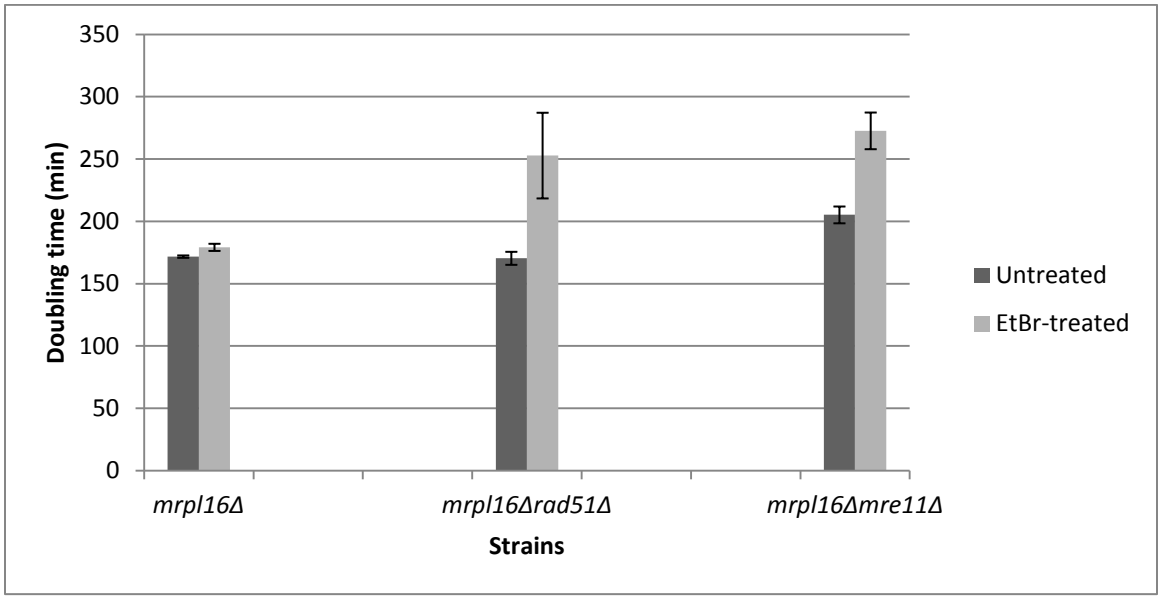


Fig 3.2 Effect of EtBr on the doubling times of DSB repair mutants in the presence and absence of mtDNA encoded products

a.



b.


350
300
250
200
150
100
50
0
Doubling time (min)
mrpl16Δ
mrpl16Δrad51Δ
mrpl16Δmre11Δ
Strains
Untreated
EtBr-treated

c.

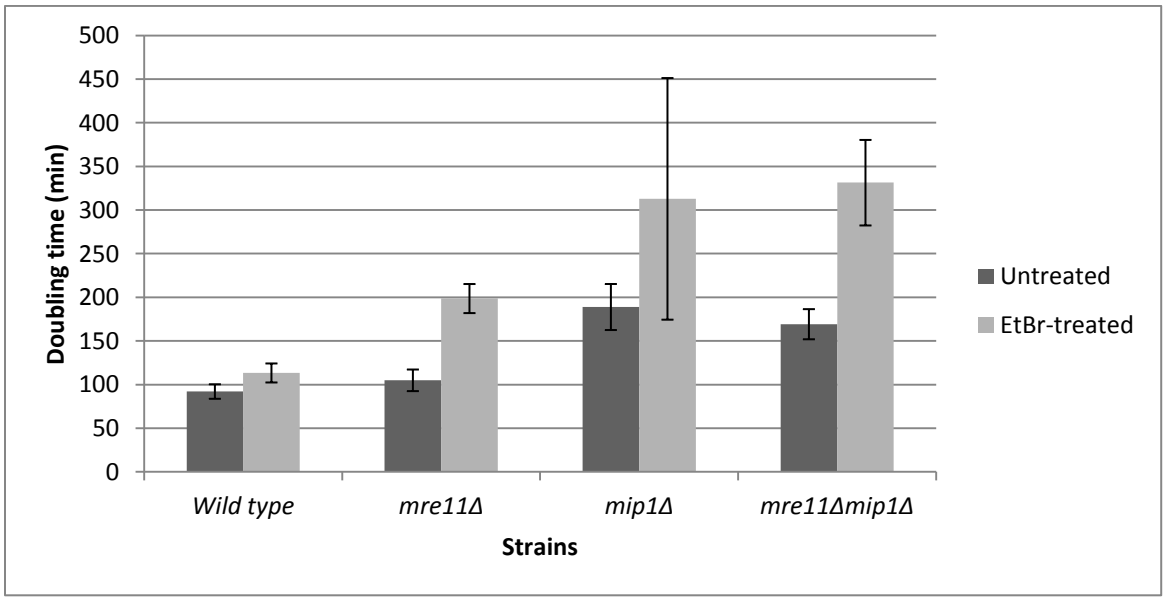

500
450
400
350
300
250
200
150
100
50
0
Doubling time (min)
Wild type
mre11Δ
mip1Δ
mre11Δmip1Δ
Strains
Untreated
EtBr-treated

Fig 3.3 A yeast model to distinguish gene conversion from mutation

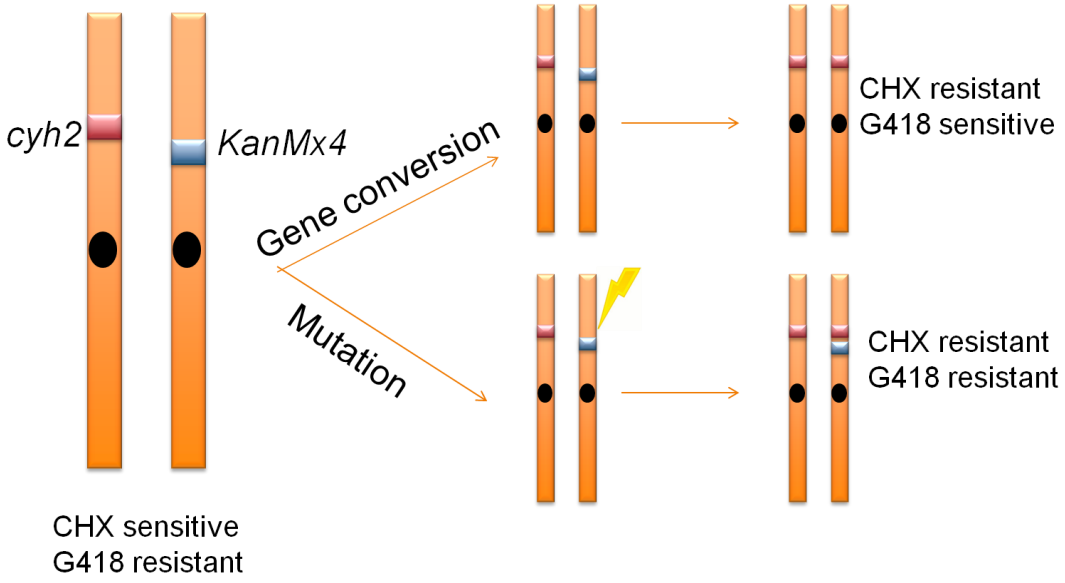
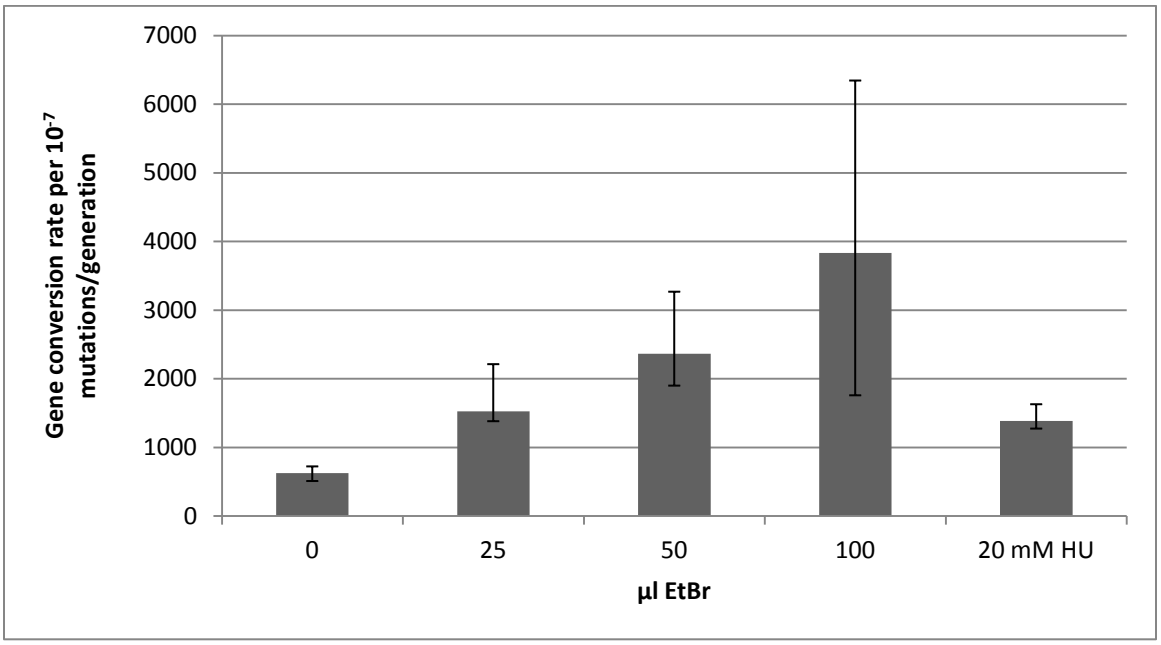


Fig 3.4 Both gene conversion rate and mutation rate increases with increasing amounts of EtBr, in the absence of mtDNA polymerase

a.



b.

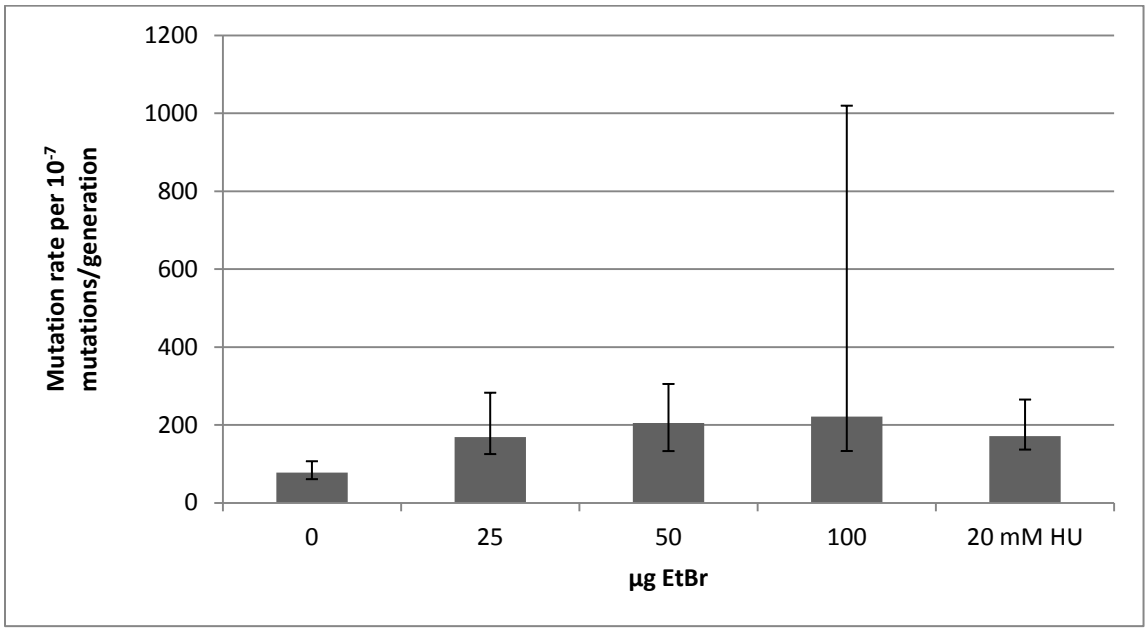
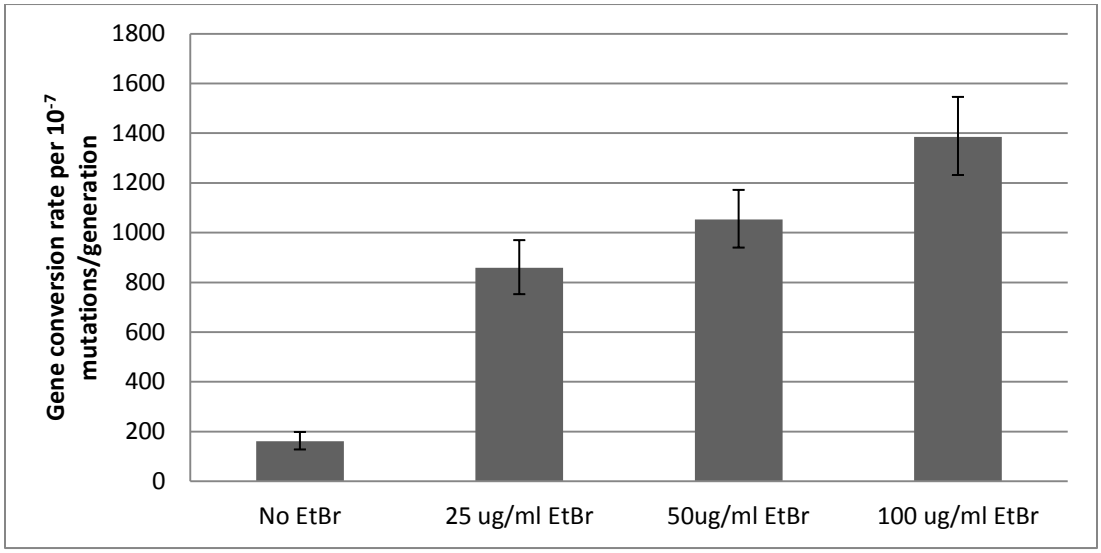

1200
1000
800
600
400
200
0
Mutation rate per 10-7 mutations/generation
0
25
50
100
20 mM HU
µg EtBr

Fig 3.5 Both gene conversion rate and mutation rate increases with increasing amounts of EtBr, in the absence of mtDNA-encoded products

a.



b.

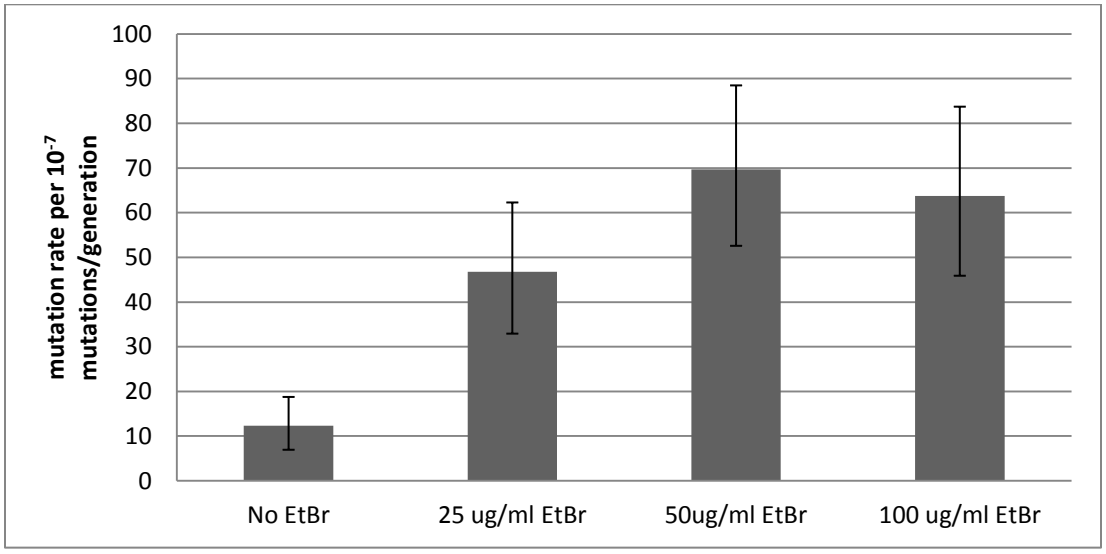
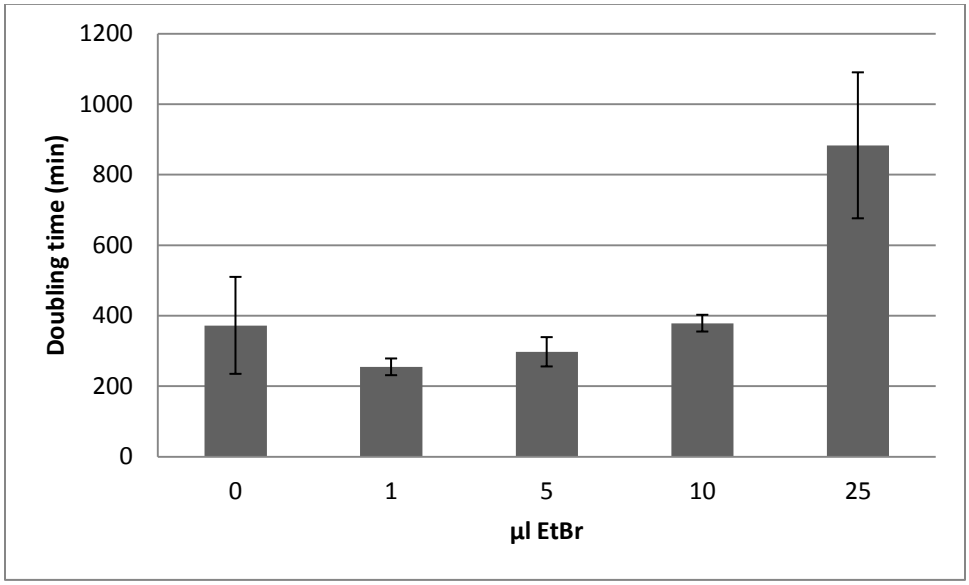


Fig 3.6 Growth of DSB mutants lacking intact mtDNA is barely affected by EtBr at concentrations less than 5 µg/ml.

a.



b.

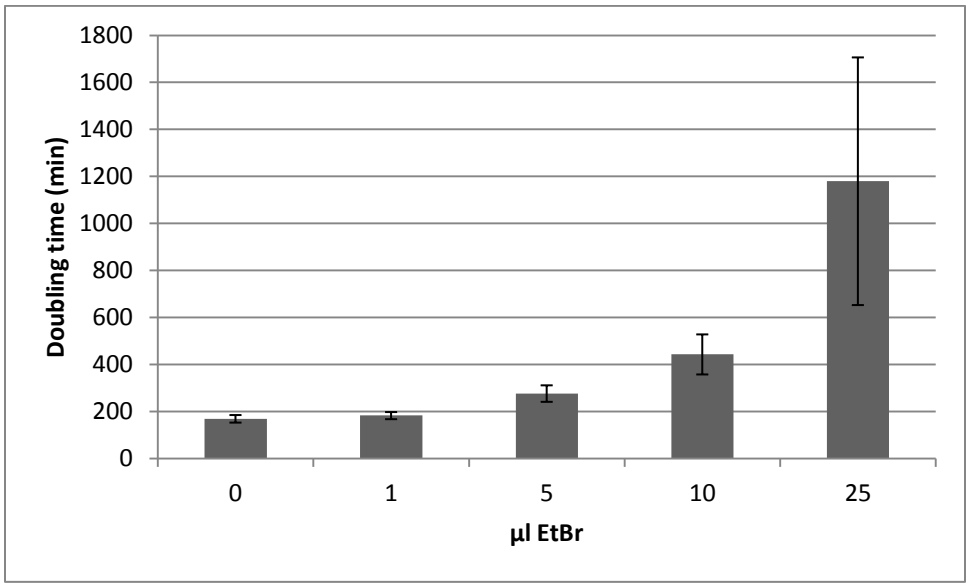

1800
1600
1400
1200
1000
800
600
400
200
0
Doubling time (min)
0
1
5
10
25
µl EtBr

Table 3.1 The mutation rates at *CAN1* locus for yeast treated with or without EtBr

	Mutation Rate (per 10^7)
Wild type	24,2223
***mrpl16*Δ -EtBr**	4,5352
***mrpl16*Δ with EtBr**	5,2360

Table 3.2 The efficacy of mtDNA loss at different EtBr concentrations

EtBr conc (µg/ml)	Relative amount of viable colonies
0	100
1	2,0714
2	0,8694
3	0,0327
4	0,0205
5	0,0173
10	0,0114
25	0,0132

b.

EtBr conc (µg/ml)	Relative amount of viable colonies
0	100
1	0,1084
5	0,0026
10	0,002
25	0,0029
50	0,008

Table 3.3 List of the strains used in this study

Strain	Genotype	Source	Parental strain(s)	Method Used
BY4741	*MATa leu2Δ0 met15Δ0 ura3Δ0 his3Δ1*	Brachman et al. (1998)		
BY4742	*MATα leu2Δ0 met15Δ0 ura3Δ0 his3Δ1*	Brachman et al. (1998)		
CDD51	*MATα leu2-3,112 ura3-1 his3-11,15 trp1Δ2 can1-100 cyh2*	Garipler at al. (2013)		
CDD65	*MATa leu2Δ0 met15Δ0 ura3Δ0 his3Δ200 trp1Δ63 cyh2*	This study	BY4733	transformed with cyh2 from CDD51
CDD68	*MATa aac2Δ::URA3 CYH2*	Garipler at al. (2013)		
CDD262	*MATα yer095wΔ::KanMX4*	EUROSCARF Y16401		
CDD263	*MATα ymr224cΔ::KanMX4*	EUROSCARF Y10810		
CDD307	*MATa/α leu2Δ0/leu2Δ0 lys2Δ0/LYS2 met15Δ0/MET15 ura3Δ0/ura3Δ0 his3Δ1/his3Δ1 ydl134cΔ::URA3/YDL134C ydl188cΔ::KanMX4/YDL188C*	Garipler at al. (2013)		
CDD409	*MATa mrpl16Δ::URA3*	This study	1854	replaced *MRPL16* using primers 260/261 and pRS306 template followed by sporulation
CDD429	*MATα mrpl16Δ::URA3 met- lys-*	This study		Crossed CDD263 and CDD409 followed by sporulation
CDD430	*MATa rad51Δ::KanMX4 mrpl16Δ::URA3 met- lys-*	This study		Crossed CDD262 and CDD409 followed by sporulation
CDD431	*MATα mre11Δ::KanMX4 mrpl16Δ::URA3 met- lys-*	This study		Crossed CDD262 and CDD409 followed by sporulation
CDD442	*MATα ygl104cΔ::KanMX4*	EUROSCARF Y14471		
CDD474	*MATα mrpl16Δ::LEU2 met- lys-*	This study	CDD307	replaced *MRPL16* using primers

				260/261 and pRS305 template followed by sporulation
CDD501	*MATα mrpl16Δ::URA3 met+ lys- trp+*	This study	BY4742 and CDD409	crossed BY4742 and CDD409 followed by sporulation
CDD502	*MATa/α mrpl16Δ::LEU2/mrpl16Δ::URA3 cyh2/CYH2 vps73Δ::KanMX4/VPS73*	This study	CDD 442,CDD409, CDD65,CDD474	crossed a spore from CDD442xCDD409 with a spore from CDD65xCDD474
CDD622	*MATα mip1Δ::URA3 lys+ met+*	This study	BY4741 and CDD263	crossed BY4741 and CDD263, then knocked out *MIP1* using primers 434/435 and PRS306 template follwed by sporulation
CDD624	*MATα mre11Δ::KanMX4 mip1Δ::URA3 lys- met+*	This study	BY4741 and CDD263	crossed BY4741 and CDD263, then knocked out *MIP1* using primers 434/435 and PRS306 template follwed by sporulation
CDD641	*MATa/α mip1Δ::URA3/mip1Δ::URA3 vps73Δ::KanMX4/cyh2 met15Δ/MET15 LYS2/LYS2 trp1Δ/TRP1*	This study	CDD65, CDD622, CDD 442, CDD623	crossed a spore from CDD442xCDD623 with a spore from CDD65xCDD622

Table 3.4 List of primers used in this study

Number	Primer Name	Sequence
260	mrpl16delfwd	TACCTTTCACCAGAGGTACAGAGGAATAGAATTTTGCAACAGATTGTACTGAGAGTGCAC
261	mrpl16delrvs	CAATAGTTATATACATGTATACATGGTGTGTTCAATCCGTctgtgcggtatttcacaccg
434	mip1delfwd	ATGGGGATTATATGTAGTTGTTGAGCAACGAGGGACAAGTAGATTGTACTGAGAGTGCAC
435	mip1delrvs	TATATATAAATACAAATGCGAAAGCTAATGCAGATTTTGCCTGTGCGGTATTTCACACCG

Chapter 4

A Novel Screen to Identify Unknown Components of Mitochondrial Fission Machinery in Budding Yeast

4.2 Introduction

Mitochondria are dynamics organelles that divide and fuse continuously. There is a balance between fusion and fission of mitochondria [5]. Mitochondria can be seen as tubular structures under the microscope when the mitochondrial fusion and fission are in balance. When the balance is shifted in favor of either process mitochondrial shape is distorted. Defects in fusion result in small, fragmented DNA networks whereas defects in fission cause mitochondria to appear as elongated, interconnected tubules. Interestingly, when both fission and fusion are inhibited, mitochondria restore their tubular shape. Defects in mitochondrial dynamics have been associated with many diseases and disorders including Parkinson's disease, optic atrophy and several types of Charcot- Marie-Tooth disease [9]. Therefore understanding the fusion and fission machineries of mitochondria is of crucial importance.

Several components of the fusion and fission machineries have already been identified. Among these, Fzo1p is a GTPase, which is essential for mitochondrial fusion[42,43]. Cells lacking Fzo1p are not able to fuse their mitochondria and therefore these cells lose the balance between fusion and fission. In order to identify a new component of fission machinery in S. cerevisiae, we designed a novel screen based on high copy suppression of *fzo1Δ*. We used a strain of S. cerevisiae that is not viable when its mitochondria are able to divide. This strain is not viable because it lacks Aac2p in addition to Fzo1p. *fzo1Δ* causes loss of mitochondrial DNA in the presence of mitochondrial fission,

while *aac2Δ* mutants cannot grow in the absence of mitochondrial DNA. This strain is transformed with a 2μ vector based high copy plasmid library. 2μ origin of replication allows the genes on the plasmid to be overexpressed. One of the possible outcomes of this over expression is over expression of a suppressor of *fzo1Δaac2Δ*. This suppression could be due to stoichiometric inhibition of the fission machinery or deregulation of the fission by interfering with the transcription, translation or degradation of a fission machinery component. If the overexpressed protein is a component of a protein complex of three or more proteins, that is functional as a complex, we would expect that it would disrupt the function of the complex by inhibiting the interactions between the other components of complex since it will form sub-stoichiometric complexes with them separately[44]. The disruption in the fission machinery will cause suppression of *fzo1Δ* by bringing fusion and fission into balance again.

The screen is as follows: The starting strain fuses mitochondria due to the wild copy of the *FZO1* gene in the plasmid it carries. Although it has a chromosomal mutant *cyh2* gene which causes the cells to be resistant to cycloheximide (a drug that inhibits cytoplasmic protein synthesis), the starting strain is sensitive to cycloheximide due to the wild type *CYH2* gene in the plasmid. The strain is transformed with a 2μ-LEU2-genomic DNA plasmid library which causes the overexpression of the gene fragment it carries. When the transformants are grown on a plate that contains cycloheximide, the cells are forced to lose the *pTRP1-FZO1-CYH2* plasmid. Since the cells lack *FZO1*, their mitochondria are not able to fuse. Since they also lack *AAC2*, the cells could survive if and only if the product of X gene, that is overexpressed by the plasmid, blocks mitochondrial fission

4.2 Results

Around 16000 colonies transformed with *URA3* library and 24000 colonies transformed with *LEU2* library were screened. I used two different libraries in order not to miss any gene fragment that is not represented in one library. Therefore 40000 colonies in total were screened. Among those 40000 colonies around 250 were shown to be grown on plates containing cycloheximide. The colonies that were cycloheximide resistant were checked if they have *FZO1* or AAC2 genes. The cells which were cycloheximide resistant ,due to the loss of *FZO1-CYH2-LEU2* or *FZO1-CYH2-URA3* plasmid, and proven to be lacking *FZO1* or *AAC2* transformed within the plasmid library were selected. These colonies were further checked for their dependence on the plasmid by being streak onto SD-Ura for *URA3* library transformed yeast and SD-Leu for *LEU2* library transformed yeast. None of them survived on media lacking those nutritional markers. Therefore we were not able to identify a gene that would suppress the *fzo1*Δ*aac2*Δ.

4.3 Materials and Methods

4.3.1 Strains, media, and genetic methods.

A list of the strains that are used in this study can be found in Table (4.1) along with their genotypes. A list of primers used in this study can be found in Table (4.2).
Diploid strain CDD58 was obtained from RJ53 by homologous recombination of the deletion cassette *aac2*Δ*::URA3/AAC2* amplified from pRS306 with primers 32 and 33. CDD63 was obtained by transforming the plasmid b1-*FZO1* to CDD59 and *aac2::URA3* in CDD63 was replaced with *aac2*Δ*::HIS3* amplified to make CDD119. CDD119 was sporulated and two *fzo1*Δ*::LEU2 aac2*Δ*::HIS3 cyh2* spores with plasmid *TRP1-FZO1-CYH2* were mated to make CDD131. CDD131 was used for screening with the 2micron based URA3 library.

Diploid strain CDD59 was obtained from CDD58 by knocking out FZO1 with LEU2 amplified with primers 30 and 31 from pRS 305. The *fzo1*Δ*::LEU2* cassette in CDD59 was replaced with *fzo1*Δ*::HIS3* cassette and the Leu^- colony was picked to obtain CDD62. CDD63 was made transforming CDD59 with b1*FZO1* isolate 2. CDD67 was obtained by sporulation of CDD63 and it was transformed with pRS313-*FZO1* and cured of b1*FZO1* isolate 2 by 10 mg/L cyloheximide. This new haploid *fzo1*Δ*::LEU2 aac2*Δ*::URA3 cyh2* pRS313-*FZO1* strain was named CDD70. After CDD62 was transformed with b1*FZO1*-4, it was sporulated and the haploids were mated to CDD70 and tested for the ability to lose plasmid b1*FZO1* and grow on cycloheximide. The resulting *fzo1*Δ*::HIS3 aac2*Δ*::URA3 cyh2* + b1FZO1 isolate4 strain was named CDD71. CDD71 was mated to CDD51 which was a W303 background cyh2 strain selected on 10 mg/L CHX. The resulting diploid *fzo1*Δ*::HIS3/FZO1 aac2*Δ*::URA3/AAC2 cyh2/cyh2* strain was named CDD202. CDD202 was sporulated and two His^+ Ura^+ *cyh2* spores from CDD202 were mated to obtain CDD270. CDD270 was used for screening with the 2 micron based *LEU2* library.Media and genetic techniques were as described (Adams *et al.*, 1997).

4.3.2 The overexpression screen.

The diploid *fzo1*Δ*::LEU2/fzo1*Δ*:LEU2 aac2*Δ*::HIS3/aac2*Δ*::HIS3 cyh2/cyh2* +pTRP1-FZo1-CYH2 strain CDD131 is transformed with the plasmid library L12. After incubating the SD-Ura plates at 30°C for 3 days they were replica plated to YEPD plates containing 10 µg/ml cycloheximide. The replicas are incubated at 30°C for 1 day whereas the original plates were stored at 4°C. The colonies that were viable on cycloheximide containing media were selected and colony purified on YEPD plates. The single colonies were picked and grown in 4 ml YEPD at 30°C overnight. Genomic DNA (gDNA) was isolated from those cultures. The primers 64 and 65 which amplify *FZO1* from the inside of the gene and the primers 62 and 63 which amplify *AAC2* from the inside of the gene were

used to detect the absence of the *FZO1* and *AAC2* genes. During these PCRs, primers 54 and 55 which amplify *FIS1* were used as controls for the PCR and gDNA extraction. The colonies lacking *FZO1* and *AAC2* were plated on SD-Ura media and further checked for the dependence on the 2µ *URA3* plasmid.

The diploid *fzo1*Δ*::URA3/fzo1*Δ*:URA3 aac2*Δ*::HIS3/aac2*Δ*::HIS3 cyh2/cyh2* +pTRP1-FZo1-CYH2 strain CDD131 is transformed with the plasmid library YEP13 (a gift from Ahmet Koç, İYTE). After incubating the SD-Leu plates at 30°C for 3 days they were replica plated to YEPD plates containing 10 µg/ml cycloheximide. The replicas are incubated at 30°C for 1 day whereas the original plates were stored at 4°C. The colonies that were viable on cycloheximide containing media were selected and colony purified on YEPD plates. The single colonies were picked and grown in 4 ml YEPD at 30°C overnight. Genomic DNA (gDNA) was isolated from those cultures. The primers 64 and 65 which amplify *FZO1* from the inside of the gene and the primers 62 and 63 which amplify *AAC2* from the inside of the gene were used to detect the absence of the *FZO1* and *AAC2* genes. During these PCRs, primers 54 and 55 were used as controls for the PCR and gDNA extraction. The colonies lacking *FZO1* and *AAC2* were plated on SD-Leu media and further checked for the dependence on the 2µ *LEU2* plasmid.

Table 4.1 Results of the screen

Total number of transformants	Around 40 000
Survived after losing *FZO1-CYH2-TRP1* plasmid	Around 200
Has *FZO1*	24
Has *AAC2*	3
Depend on the overexpression plasmid for survival	0

Table 4.2 Strains used in this study

Strain	Genotype	Source	Parental strain(s)	Method Used
CDD 51	*MATα leu2-3,112 ura3-1 his3-11,15 trp1Δ2 can1-100 cyh2*	Gariplerat al. (2013)		
CDD58	*MATa/α ura3-1/ura3-1 trp1Δ2/trp1Δ2 ade2-1/ade2-1 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 can1-100/can1-100 cyh2/CYH2 aac2Δ::URA3/AAC2*	Garipleret al. (2013)		
CDD59	*MATa/α ura3-1/ura3-1 trp1Δ2/trp1Δ2 ade2-1/ade2-1 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 can1-100/can1-100 cyh2/CYH2 aac2Δ::URA3/AAC2 ybr179cΔ::LEU2/YBR179C*	Garipleret al. (2013)		
CDD62	*MATa/α ura3-1/ura3-1 trp1Δ2/trp1Δ2 ade2-1/ade2-1 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 can1-100/can1-100 cyh2/CYH2 aac2Δ::URA3/AAC2 fzo1Δ::HIS3/FZO1*	This study	CDD59	knocked out *fzo1Δ::LEU2* with *fzo1Δ::HIS3* cassette
CDD63	*MATa/α ura3-1/ura3-1 trp1Δ2/trp1Δ2 ade2-1/ade2-1 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 can1-100/can1-100 cyh2/CYH2 aac2Δ::URA3/AAC2 ybr179cΔ::LEU2/YBR179C pTRP1-CYH2-YBR179C*	Garipleret al. (2013)		

CDD67	*MATα ura3-1 trp1Δ2 ade2-1 leu2-3,112 his3-11,15 can1-100 cyh2 aac2Δ::URA3 fzo1Δ::LEU2 pTRP1-CYH2-YBR179C*	This study	CDD63	sporulation
CDD70	*MATα ura3-1 trp1Δ2 ade2-1 leu2-3,112 his3-11,15 can1-100 cyh2 aac2Δ::URA3 fzo1Δ::LEU2 pRS313-FZO1*	This study	CDD67	transformed with pRS313-*FZO1* and cured of b1*FZO1* by 10 mg/L cycloheximide
CDD71	*MATa fzo1Δ::HIS3 aac2Δ::URA3 cyh2 pFZO1-TRP1-CYH2*	This study	CDD62, CDD70	CDD 62 transformed with b1*FZO1*, sporulated, haploids mated to CDD70 and tested for ability to lose plasmid b1*FZO1* and grow on CHX
CDD119	*MATa/α ura3-1/ura3-1 trp1Δ2/trp1Δ2 ade2-1/ade2-1 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 can1-100/can1-100 cyh2/CYH2 aac2Δ::HIS3/AAC2 fzo1Δ::LEU2/FZO1 pTRP1-CYH2-YBR179C*	This study	CDD63	replaced *aac2Δ::URA3* with *acc2Δ::HIS3*
CDD131	*MATa/α ura3-1/ura3-1 trp1Δ2/trp1Δ2 ade2-1/ade2-1 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 can1-100/can1-100 cyh2/Ccyh2 aac2Δ::HIS3/aac2Δ::HIS3 fzo1Δ::LEU2/fzo1Δ::LEU2 pTRP1-CYH2-FZO1*	This study	CDD119	spores mated
CDD202	*MATa/α fzo1Δ::HIS3/FZO1 aac2Δ::URA3/AAC2 cyh2/cyj2 pTRP1-FZO1-CYH2*	This study	CDD51 and CDD71	mating
CDD270	*MATa/α fzo1Δ::HIS3/ fzo1Δ::HIS3 aac2Δ::URA3/aac2Δ::URA3 cyh2/cyh2 pTRP1-FZO1-CYH2*	This study	CDD202	sporulation followed by mating

Table 4.3 Primers used in this study

Number	Primer Name	Sequence

54	fis1ampfwd	CAGTTCAAATAACATGTGTCCATTACCTGTAC
55	fis1amprvs	AGAAGGCAAAATAGCAGTGCGTTTATATAC
62	aac2intchkfwd	CCAAAACCAAGATGAAATGTTAAAACAAGGTACTTTGGA
63	aac2intchkrvs	CGTACATACCGAAGTATAGACCTCTGTAGAC
64	fzo1intchkfwd	GGGACAAACAACGTTGTAAGGAGTTGATCCTAAAGCA
65	fzo1intchkrvs	GTATTGTTATAAGTCATTGTAATTGTTTGCTCGGC

Appendix

A Novel Method to Measure Recombination Rate in Yeast Using Counter-Selection

5.1 Introduction

Chemicals such as pharmaceuticals could cause damage in the genome of organisms in the form mutations, recombinations or chromosomal aberrations. It is important to detect this so called "genotoxic" effects of especially the pharmaceuticals before they get commercialized. The already available methods to detect the effects of chemicals on genome are limited. They either detect mutations at the single nucleotide level or they detect gross chromosomal rearrangements. The classic Ames test is an in vitro assay that is used to detect if a chemical causes mutations in the genome of bacteria. Chromosomal aberration test and micronuclei assay are used to detect gross chromosomal rearrangements. However, these assays detect gross rearrangements that could be seen only with a microscope. Therefore, there is a need for a new genotoxicity test that will allow us to detect the changes in the genome such as recombination, which is bigger than a mutation but too small to be detected under the microscope.

5.2 A One-Step Procedure to Detect Recombination

We designed a strain of yeast which could be used to detect mitotic recombination. Our diploid strain has a recessive *cyh2* allele in one of the chromosomes. The most tightly linked gene to *CYH2* is *VPS73* in yeast genome. The strain has a *URA3* marker instead of *VPS73 in trans* to the *cyh2* allele. The *URA3* marker gives us the ability to both positive and negative selection. The negative selection is done using 5-FOA which is converted to toxic 5-flurouracil in the presence of *URA3* gene product. Therefore, if our strain gains resistance to cycloheximide through mutation, the *URA3* gene won't be affected and the

strain won't be able to live on media containing 5-FOA. However, if a recombination of the *cyh2* allele causes the yeast to become resistant to CHX, then the *URA3* gene will be lost during the recombination and the yeast will become resistant to 5-FOA, too. Therefore, the recombination rate could be calculated from the number of colonies that are resistant to both CHX and 5-FOA using fluctuation analysis.

We tested the sensitivity of the strain using HU which is known to increase genomic instability. Our first results were promising. We were able to detect around 6 fold increase in the genomic instability of the strain upon treatment with 20mM HU for 15 generations (Table 5.1). However, when we created the same strain in a different background we were not able to detect any significant change in genomic instability upon HU treatment. (Table 5.2).

As the strain that we used at Chapter 3 to detect recombination did not detect a dramatic change in recombination after HU treatment despite having worked for EtBr, our system might be only valid for the mechanism of EtBr. Therefore, this method might still be used to detect genomic instability but the chemicals that could be identified as genomic instability causing agent might be limited.

Table 5.1 Effect of HU on gene conversion rate of the strain with counterselectable marker in BY background

Treatment	Gene conversion rate ($x10^7$)
Untreated	130,4522
Treated with HU	983,7963

Table 5.2 Effect of HU on the gene conversion rate of the strain with counterselectable marker in W303 background

Untreated	Treated with 20 mM HU
Gene Conversion Rate ($x10^7$)	**Gene Conversion Rate** ($x10^7$)
812,7361	626,1080
Single Mutation Rate (M) ($x10^7$)	**Single Mutation Rate (M)** ($x10^7$)
70,8220	46,2679

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